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(54) **Multiply mutated subtilisins**

Mehrfach mutierte Subtilisine

Subtilisines à mutations multiples

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Description

The present invention provides a novel class of thermally stable and pH stable subtilisin analogs with improved oxidation stability and superior performance in cleaning soiled fabrics when added to a standard detergent formulation, and to a method for preparing such analogs. In particular, the present invention relates to a class of subtilisin analogs having a modified calcium binding site providing improved calcium binding capacity, and a deletion and/or replacement of either residue of Asn-Gly sequences present in the subtilisin and a modification of one or more methionine residues to improve oxidation stability. The present invention further relates to detergent compositions containing such subtilisins .

Background of the Invention

The term subtilisin designates a group of extracellular alkaline serine proteases produced by various species of Bacilli. These enzymes are also referred to as Bacillus serine proteases, Bacillus subtilisins or bacterial alkaline proteases.

Bacillus subtilisin molecules are composed of a single polypeptide chain of either 274 residues (for subtilisin type Carlsberg produced by Bacillus licheniformis and for the subtilisin produced by Bacillus subtilis strain DY) or 275 residues (for subtilisin type BPN′ produced by Bacillus amyloliquefaciens, the aprA gene product of Bacillus subtilis, and the subtilisin of Bacillus mesentericus and the subtilisin of Bacillus subtilis var. amylosacchariticus). When comparing amino acid sequences of subtilisin from different strains of Bacillus herein, the sequence of subtilisin BPN′ is used as a standard. For example, based on an alignment of sequences that gives the highest degree of homology between subtilisin Carlsberg and subtilisin BPN′, the serine at the active site of the former is referred to as serine 221, even though it is located at position 220 of the amino acid sequence. On the same basis, position 220 of the amino acid sequence of subtilisin Carlsberg may be said to "correspond" to position 221 of subtilisin BPN′. See e.g., Nedkov et al., Hoppe-Seyler's Z. Physiol. Chem., 364, 1537-1540 (1983).

The X-ray structure of subtilisin BPN′ [Wright, et al., Nature, 221, 235 (1969)] revealed that the geometry of the catalytic site of subtilisin, involving Asp^{32}, His^{64} and Ser^{221}, is almost identical to that of the active site of mammalian serine proteases (e.g., chymotrypsin) involving the residues Asp^{102} His^{57}, and Ser^{195}. However, the overall dissimilarities between Bacillus serine proteases and mammalian serine proteases indicate that these are two unrelated families of proteolytic enzymes.

In the family of Bacillus subtilisins complete amino acid sequences are available for six subtilisins: Carlsberg, [Smith, et al., J. Biol. Chem., 243, 2184-2191 (1968)]; BPN′ [Markland, et al., J. Biol. Chem., 242, 5198-5211 (1967)]; the aprA gene product [Stahl, et al., J. Bacteriol., 158, 411-418 (1984)]; DY [Nedkov, et al., supra]; Bacillus mesentericus [Svendsen, et al., FEBS Letters, 196, 220-232 (1986), and Bacillus subtilis var. amylosacchariticus [Yoshimoto, et al., J. Biochem., 103, 1060-1065 (1988)]. Subtilisin Carlsberg and subtilisin BPN′ (sometimes referred to as subtilisin Novo) differ by 84 amino acids and one additional residue in BPN′ (subtilisin Carlsberg lacks an amino acid residue corresponding to residue 56 of subtilisin BPN′). Subtilisin DY comprises 274 amino acids and differs from subtilisin Carlsberg in 32 amino acid positions and from subtilisin BPN′ by 82 amino acid replacements and one deletion (subtilisin DY lacks an amino acid residue corresponding to residue 56 of subtilisin BPN′). The amino acid sequence of the aprA gene product is 85% homologous to the amino acid sequence of subtilisin BPN′. Thus, it appears that there is an extensive homology between amino acid sequences of subtilisins from different strains of Bacillus. This homology is complete in certain regions of the molecule and especially in those that play a role in the catalytic mechanism and in substrate binding. Examples of such sequence invariances are the primary and secondary substrate binding sites, Ser^{125}-Leu^{126}-Gly^{127}-Gly^{128} and Tyr^{104} respectively and the sequence around the reactive serine (221), Asn^{218}-Gly^{219}-Thr^{220}-Ser^{221}-Met^{222}-Ala^{223}.

Subtilisin molecules exhibit unique stability properties. Although they are not completely stable over a wide pH range, subtilisins are relatively resistant to denaturation by urea and guanidine solutions and their enzymatic activity is retained for some time in 8 M urea. In solutions having a pH below 4, subtilisin rapidly and irreversibly loses its proteolytic activity. Gounaris, et al., Compt. Rend. Trav. Lab. Carlsberg, 35, 37 (1965) demonstrated that the acid deactivation of subtilisin is not due to a general charge effect and speculated that it is due to other changes in the molecule, such as protonation of histidine residues in the interior, hydrophobic parts of the molecule. Bacillus subtilisins undergo irreversible inactivation in aqueous solutions at a rate that is largely dependent upon temperature and pH. At pH values below 4 or above 11 the rate of inactivation is very rapid while at pH's of between 4.5 and 10.5 the rate, although much slower, increases as the solution becomes more alkaline. The mechanisms of this inactivation are not fully known but there is evidence indicating that autodigestion is responsible at least in part for enzyme instability at this pH range. In general, at any pH value, the higher the temperature the faster the rate of subtilisin deactivation.

The use of proteases in industrial processes which require hydrolysis of proteins has been limited due to enzyme instability under operational conditions. Thus, for example, the incorporation of trypsin into laundry detergents (e.g.,

Bio-38, Schnyder; Switzerland) to facilitate removal of proteinaceous stains had a very limited success which was undoubtedly a result of enzyme instability under the washing conditions. In addition, bacterial alkaline proteases compatible with detergents have been utilized in detergent formulations.

Because many industrial processes are conducted at temperatures that are above the stability range of most enzymes, highly thermostable proteases not only will be advantageous to certain industries such as detergent and hide dehairing, that already require stable proteases, but may be useful in industries that use chemical means to hydrolyze proteins e.g. hydrolysis of vegetable and animal proteins for the production of soup concentrates.

Although thermal inactivation may be the most important factor in restricting the industrial use of enzymes, other factors such as need for effectiveness over broad pH ranges and use of denaturing agents and oxidizing agents may also have a detrimental effect with respect to the use of proteases in industrial processes. It is therefore desirable to obtain a class of proteases characterized by improved stability with respect to temperature, pH, denaturing agents and oxidizing agents and other conditions required by various industries.

Over the past several years there have been major changes in detergent formulations, particularly in the replacement of phosphates with alternate builders and in the development of liquid laundry detergents to meet environmental and consumer demands. These changes create a need for changes in traditional detergent enzymes. More particularly, it has become desirable to employ proteolytic enzymes which possess greater storage stability in liquid laundry formulations as well as stability and activity at broader ranges of pH and temperature in a variety of commercial detergent formulations.

One approach to producing modified subtilisins useful in detergent formulations was disclosed in U.S. Patent No. 4,760,025 wherein mutations in the subtilisin of Bacillus amyloliquefaciens (B. amyloliquefaciens) at positions Asp^{32}, Asn^{155}, Tyr^{104}, Met^{222}, Gly^{166}, His^{64}, Gly^{169}, Phe^{189}, Ser^{33}, Tyr^{217}, and/or Gly^{157} were identified as providing changed stability, altered conformation or as having changes in the "processing" of the enzyme. In particular, a mutation of Met^{222} to Ala or Ser assertedly resulted in improved oxidation stability, but the specific activity of the enzyme toward the synthetic peptide substrate, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-p-nitroanilide (sAAPFpN) was decreased when compared to the unmutated enzyme. It was suggested that substitution for Gly^{166} with Ala, Asp, Glu, Phe, His, Lys, Asn, Arg or Val would alter the kinetic parameters of the enzyme. However, none of the mutations disclosed provide analogs having greater stability at high temperatures or stability over a broader pH range than the wild type enzyme.

In another approach, Thomas, et al, Nature, 318, 375-376 (1985), disclosed that the pH dependence of subtilisin may be altered by changing an Asp to Ser in Asp^{99} of subtilisin BPN'. This change represents an alteration of a surface charge 14-15 Angstroms from the active site. However, Thomas, et al. fails to provide any indication of improvement where no change in surface charge is made, as is the case where one uncharged residue is substituted for another.

A third approach, described in WO87/04461 and WO 88/08033 relates to a class of Bacillus serine protease analogs characterized by deletion and/or modifications of any Asn-Gly sequences present in the protease.

Takagi et al., J. Biol. Chem. 263, 19592-19596 (1988) disclose that a change of isoleucine 31 to leucine increases the activity of subtilisin compared to the wild-type enzyme.

Summary of the Invention

The present invention provides a class of subtilisin analogs characterized as having improved pH and thermal stability and oxidation stability thereby rendering such analogs especially useful in detergent formulations as well as other processes requiring stable proteases. The subtilisin analogs according to the present invention are characterized as having an amino acid sequence of a naturally occurring Bacillus subtilisin that has been modified.

The invention provides a substantially pure [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin analog, optionally with one additional substitution selected from [Leu^{31}] and [Leu^{124}]. The present invention further provides detergent compositions comprising the subtilisin analogs of the present invention and to the use of such subtilisin analogs and compositions in cleaning applications.

The subtilisin analogs of the present invention exhibit improved thermal, pH stability and oxidation stability, increased specific activity and broad substrate specificity thereby increasing the detergency of detergent formulations containing such analogs. In particular, the subtilisin analogs of the present invention provide improved thermostability, increased pH stability, increased oxidation stability and higher specific activity than found in "wild type" subtilisins.

There is disclosed herein a process for the production of subtilisin analogs comprising a host cell having nucleic acid encoding a subtilisin analog as described above. In such a cell, the nucleic acid encoding the subtilisin analog may be chromosomal or extrachromosomal. The host cell is preferably selected from a strain deficient in secreted proteases, allowing for facile isolation of the analogs of the present invention.

Brief Description of the Drawings

Fig. 1 schematically illustrates the cyclization of Asn-Gly residues, such as those found at positions 218 and 219 of subtilisin as set forth in Table 1, to form anhydroaspartylglycine and also depicts base-catalyzed hydrolysis thereof;

Fig. 2 is a partial restriction map of an aprA gene-containing an EcoRI-KpnI gene fragment of Bacillus subtilis (B. subtilis) strain QB127 and includes a partial restriction map of the aprA gene and flanking sequences;

Fig. 3 is a partial restriction map of a plasmid pAMB11;

Fig. 4 is a flowchart illustrating stages in construction of pAMB113, a plasmid which directs synthesis of [Ser]^{218}-subtilisin from B. subtilis host cells;

Fig. 5 is a partial restriction map of pAMB30 plasmid;

Fig. 6 illustrates the construction of pAMB106;

Fig. 7 illustrates the construction of M13 mp19 aprA143.

Fig. 8 shows the stability of [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin in bleach.

Figures 9 and 10 show the washing performance of [Asp^{76}, Ser^{109} Ser^{218}, Ala^{222}] subtilisin.

Detailed Description of the Invention

The term "subtilisin" as employed herein, refers to a mature, secreted form of the enzyme which lacks leader sequences cleaved from the mature enzyme prior to or at secretion. Subtilisins that may be modified in accordance with the present invention include, but are not limited to, naturally occurring subtilisins represented by the amino acid sequence of subtilisin Carlsberg, subtilisin BPN', the aprA gene product of Bacillus subtilis, subtilisin DY and the subtilisin of Bacillus mesentericus, and the subtilisin of B. subtilis var. Amylosacchariticus. The amino acid sequence for subtilisin Carlsberg is described by Smith, et al., J. Biol. Chem., 243, 2184-2191 (1968). The amino acid sequence for subtilisin BPN' is described by Markland, et al., J. Biol. Chem., 242, 5198-5211 (1967). The amino acid sequence for subtilisin DY is described by Nedlov, et al., Hoppe-Seyler's Z. Physiol. Chem., 364, 1537-1540 (1983). The amino acid sequence for the subtilisin of Bacillus mesentericus is described by Svedsen, et al., FEBS Letters, 196, 220-232 (1986). The amino acid sequence for the subtilisin of Bacillus is described by Yoshimoto, et al., J. Biochem. subtilis var. amylosacchariticus, 103, 1060-1065 (1988). The amino acid sequence of the aprA gene product of Bacillus subtilis is described by Stahl, et al., J. Bacteriol., 158, 411-418 (1984). The amino acid sequence of such subtilisins are incorporated by reference herein. Such subtilisins are characterized as having calcium binding sites necessary to stabilize the molecule.

In accordance with the present invention, a class of subtilisin analogs are provided which possess improved capacity to bind to calcium. Calcium has been used to stabilize subtilisin in powders and liquid detergent, especially in applications requiring higher temperatures. The present invention relates to the modification of the calcium binding site of the subtilisin molecule to increase calcium binding. As used herein the term "modification of the calcium binding site" refers to replacement of one or more amino acids in the region of a calcium binding site present in the amino acid sequence of subtilisin with a negatively charged amino acid thereby enabling the resulting subtilisin analog to have an additional negative charge. It has been found that one calcium binding site in a subtilisin involves the following amino acids: Gln2, Asp^{41}, Leu^{75}, Asn^{76}, Asn^{77}, Ser^{78}, Ile^{79}, Gly^{80}, Val^{81}, Thr^{208} and Tyr^{214} relative to the amino acid sequence set forth in Table 1.

One preferred embodiment of the present invention involves a subtilisin analog wherein Asn^{76} is is converted to Asp^{76} and substitutions of Asn^{109} and Asn^{218} to Ser^{109} and Ser^{218}, thus eliminating two unstable Asn-Gly sequences, in combination with change of methionine 222 to alanine. Another preferred embodiment involves the above preferred modifications to the calcium binding site and Asn-Gly sequences and Met-222 in combination with amino acid modifications that increase the specificity of subtilisin to an azocasein substrate or a sAAPFpN substrate i.e., Methionine 124 to Leucine or Alanine and/or Isoleucine 31 to Leucine.

In addition, as previously noted, a class of analogs of a Bacillus subtilisin according to the present invention have an amino acid sequence wherein in addition to modifications at the calcium binding site(s) and Asn-Gly sequences, a Met residue at position 222 is replaced with Ala, to improve the oxidation stability of the subtilisin analog.

The specific activity of an analog incorporating the Met^{222} to Ala substitution can be increased by incorporating one or more of the following additional changes: Met^{124} to Leu, Ile^{31} to Leu.

Due to their capacity to secrete substantial quantities of proteins and because they are currently used to produce detergent proteases, Bacillus microorganisms represent a preferred host for recombinant production of the subtilisin analogs according to the present invention. Because most Bacilli secrete alkaline and neutral proteases, it is preferable that mutations be introduced into the endogenous alkaline and neutral protease genes of B. subtilis so that the mutated subtilisin may be produced and secreted by B. subtilis in a medium free of other proteases. Thus the present invention also provides mutant strains of B. subtilis which are blocked with respect to the synthesis of endogenous proteases

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but which retain the ability to synthesize and secrete the subtilisin analogs herein disclosed.

As described in greater detail below, it was found that the pH and thermal stability and oxidation stability and the stability in detergent formulations of the subtilisin analogs of the present invention is significantly greater than that of the wild type aprA gene product subtilisin and Carlsberg subtilisin.

All subtilisin analogs according to the invention may be prepared in accordance with the following procedure:

1) Isolation of the representative subtilisin gene aprA from B. subtilis;
2) Cloning of the aprA gene on a vector which permits utilization of oligonucleotide site-directed mutagenesis to create desired modifications;
3) Site-directed mutagenesis and sequencing of the resulting DNA to confirm the presence of the desired mutation;
4) Construction of an expression vector to direct the synthesis of the mutated enzyme in B. subtilis;
5) Construction of mutated B. subtilis strains which do not synthesize subtilisin and neutral protease;
6) Isolation of the enzyme in the extra-cellular growth medium and its purification;
7) Practice of procedures for insertion of the gene coding for the improved enzyme into the chromosome of a B. subtilis strain previously mutated to block synthesis of endogenous proteases.

As used herein, the specific subtilisin analogs are indicated by representing the replacement amino acid in brackets. For example, a [Ser^{109}] subtilisin refers to a subtilisin molecule having a serine in amino acid position 109 and a [Ser^{109}, Ser^{218}] subtilisin refers to a subtilisin molecule having a serine at amino acid positions 109 and 218.

In Example 1, the aprA gene encoding subtilisin is isolated from the B. subtilis genome. In Example 2, the aprA gene is subjected to site-directed mutagenesis. In Example 3, an expression vector containing the mutated aprA gene is constructed. In Example 4, a [Ser^{109}] subtilisin analog is prepared. Example 5 describes the preparation of a [Ser^{109}, Ser^{218}] subtilisin analog. Example 6 describes preparation of a [Asp^{76}, Ser^{109}, Ser^{218}] subtilisin analog. Example 7 describes the preparation of a [Asp^{76}, Glu^{79}, Ser^{109}, Ser^{218}] subtilisin analog.

In Example 8, a [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin analog is prepared. In Example 9, the [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin analog gene is transferred to bacteriophage M13mp19 in preparation for site-directed mutagenesis. In Example 10, a [Leu^{31}, Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin analog is prepared. Example 11 describes the preparation of a [Asp^{76}, Ser^{109}, Leu^{124}, Ser^{218}, Ala^{222}] subtilisin analog.

In Example 12, two mutant strains of B. subtilis which produce no detectable extracellular proteases are constructed. Example 13 describes procedures for integration of a mutated aprA gene into the chromosome of B. subtilis. In Example 14, wild-type and mutant aprA subtilisins are isolated and purified. Examples 15, 16, 17, 18, and 19 describe characteristics of the subtilisin analogs of the present invention regarding stability, activity and washing performance.

In addition to a subtilisin analog of the present invention, detergent compositions of the present invention may comprise:

(a) At least one surfactant which may be anionic, non-ionic, or amphoteric, or a water-soluble soap. Typically, an anionic surfactant (e.g., a linear alkyl aryl sulphonate) is used in admixture with a non-ionic (e.g., an alkyl phenyl polyglycol ether) in amounts of 5-30 and 1-5 percent by weight, respectively, of the detergent composition.
(b) One or more builders, preferably having a concomitant sequestering function. Sodium tripolyphosphate, sodium citrate, sodium silicate, and zeolites are examples of such compounds, usually constituting from 10 to 70 percent by weight of the detergent composition.
(c) A bleaching agent, preferably a peroxy compound such as sodium perborate, typically incorporated in an amount up to 30 percent by weight of the composition.
(d) Ancillary agents, such as carboxymethyl cellulose, optical brighteners and perfumes. If required, a pH-adjusting agent is added to give a pH of the laundering medium in the range of from 7.0 to 10.5.

The detergent compositions contain an effective amount of one or more of the subtilisin analogs of the present invention. As used herein "effective amount of a subtilisin analog" refers to the quantity of subtilisin analog necessary to achieve the enzymatic activity necessary in the specific detergent composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the particular subtilisin analog utilized, the cleaning application, the specific composition of the detergent composition, whether a liquid or dry composition is required and the like.

The particulate subtilisin analog preparation of the invention is added in an amount calculated to give an enzyme activity of at least 0.1 Anson units (AU, vide infra), preferably 0.5-2.5 AU per 100 g of detergent composition. If required, balance to 100 percent may be established with an inorganic filler, preferably sodium sulphate.

Liquid detergent compositions may be prepared from enzyme slurries, preferably in non-aqueous media. Typically, such slurries may consist of a suspension of finely ground subtilisin analog concentrate in a liquid non-ionic surfactant, for example Tergitol 15 S 9 or a mixture of such surfactants. Usually, the slurry will also contain one or more inorganic

fillers, such as finely ground sodium chloride, optionally in admixture with a suspension stabilizer, for example fumed silica (Aerosil 200). Tergitol and Aerosil are trademarks.

A subtilisin analog of the invention is added in an amount calculated to give a protease activity of at least 0.1 AU preferably 0.5-2.5 AU per 100 g of liquid detergent composition.

The detergent compositions may be prepared in the usual manner, for example by mixing together the components. Alternatively, a pre-mix is made, which is then mixed with the remaining ingredients.

Because of the good stability and activity properties described, the subtilisin analogs according to the invention can be used in all fields where proteolytic enzymes are generally used. In particular, it can be used for detergents and cleansers or spot removers, as a depilatory in tanning, and also in the food industry for the preparation of protein hydrolysates and in serology for the detection of incomplete antibodies. It is particularly advantageous for use in the food industry and in serology that the subtilisin analogs according to the invention have excellent stability in the solid or dissolved form that physiologically acceptable quantities of calcium ions may not be necessary to stabilize the subtilisin analog in aqueous solutions, in contrast to those of other enzyme preparations.

The following Examples will further serve to illustrate the invention and show how the same may be carried into effect. Examples 10, 11, 18 and 19 are particularly important to the claimed invention.

Example 1

B. subtilis strain QB127 (trpC2 leuA8 sacU^{h}200) [Lepesant, et al., Molec. Gen. Genet., 118, 135-160 (1982)] was obtained from the Bacillus Genetic Stock Center at the Ohio State University, Columbus, Ohio. This strain overproduces extracellular serine and metal proteases, α-amylase and levansucrase relative to isogenic sacU$^+$ strains due to the pleiotropic effect of the sacU^{h}200 mutation [Lepesant, et al., in Schlessinger, D., ed., Microbiology, 1976, American Society for Microbiology, Washington, D.C., p. 65 (1976)]. Thus, strain QB127 is a suitable source of DNA for isolating the aprA gene which codes for subtilisin.

Genomic DNA was isolated from cells of B. subtilis strain QB127 in accordance with the procedure of Saito, et al., Biochim. Biophys. Acta. 72, 619-629 (1963). Purified chromosomal DNA was digested to completion with the EcoRI restriction endonuclease.

The resulting DNA fragments were resolved on a low-melting point agarose gel by electrophoresis and fragments in the 4.4 to 8.0 kilobase (kb) range were isolated. These fragments were ligated to pCFM936 (A.T.C.C. No. 53,413 from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland) an Escherichia coli (E. coli) plasmid which displays higher copy numbers at elevated temperatures and which confers kanamycin resistance. The vector was digested with EcoRI and dephosphorylated with calf intestine alkaline phosphatase prior to ligation.

The ligation products were introduced into E. coli C600 (A.T.C.C. No. 23724 from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland) and following overnight incubation on L-agar supplemented with 10 µg/ml kanamycin, kanamycin-resistant host cells were selected. Plasmid DNA was amplified by incubating the selected host cells at 42°C for 4 hours. Colonies were then transferred to nitrocellulose filters and processed in accordance with a colony hybridization procedure described by Grunstein, et al., Proc. Natl. Acad. Sci. (USA), 72, 3961 (1975).

An oligonucleotide probe was used to screen for colonies which harbored the subtilisin gene on pCFM936. The probe synthesized by the phosphite method described by Beaucage, et al., Tetrahedron Letters, 22, 1859-1862 (1981) had the nucleotide sequence

5' GCGCAATCTGTTCCTTATGGC 3'

which corresponds to the amino-terminus of the aprA gene product (Wong, et al., Proc. Natl. Acad. Sci. (USA), 81, 1184-1188 (1984); Stahl, et al., J. Bacteriol., 158, 411-418 (1984). A hybridization temperature of 55°C was employed and 5 positive colonies were identified out of a total of 400. The plasmid DNA from one of the positive colonies was designated pCFM936 apr2.

Plasmid pCFM936 apr2 was digested with EcoRI alone, with HindIII alone and with EcoRI and HindIII in combination. Sizes of EcoRI fragments of the subtilisin gene conformed to those described in Stahl, et al., supra, but several otherwise undescribed HindIII sites were discovered. As described herein in Example 3, two of the HindIII sites were utilized in the genetic manipulations of the subtilisin gene.

It was determined that a large 6.5 kb EcoRI fragment of B. subtilis QB127 genomic DNA carried the aprA gene, its regulatory sequences and unrelated flanking sequences by verifying that restriction enzyme digests conformed to the results reported by Stahl, et al., supra. This was confirmed by DNA sequencing using the dideoxy chain termination method described by Sanger, et al., J. Mol. Biol., 143, 161-178 (1980). A 3.0 kb EcoRI to KpnI subfragment of the 6.5 kb EcoRI fragment, as illustrated in Fig. 2, was also found to contain the aprA gene, its regulatory sequences, and unrelated flanking sequences. Although the KpnI-EcoRI fragment is reported to be 2.5 kb in length by Stahl, et al., and in the legend to Fig. 1 therein, comparison of the scale of Fig. 1 and the scaled depiction of the fragment therein reveal

that, even in Stahl, et al., the KpnI-EcoRI fragment is substantially larger than 2.5 kb.

A cloning vector for Bacillus host systems, plasmid pAMB11, was constructed as follows. The plasmid pTG402 (Northern Regional Research Laboratories, United States Department of Agriculture, Peoria, Illinois, strain number NRRL B-15264) was partially digested with the RsaI restriction endonuclease. Fragments were ligated to M13 mp18
5   (available from Bethesda Research Laboratories, Gaithersburg, Maryland as catalog number 8227SA) which had been previously digested with HincII. Ligation products were introduced into E. coli JM103 (available from Pharmacia, Inc., Piscataway, New Jersey as catalog number 27-1545-01) by transformation in accordance with the procedure of Mandel, et al., J. Mol. Biol., 53, 154, (1970). Bacteriophage plaques were sprayed with 0.5M catechol (prepared in distilled water) to detect the functional expression of an xylE gene derived from pTG402. The xylE gene encodes catechol
10   2,3-dioxygenase and is useful for detecting promoters in a variety of organisms [Zukowski, et al., Proc. Natl. Acad. Sci. (USA), 80, 1101-1105 (1983)].

The xylE gene was then transferred as a 1.0 kb EcoRI to PstI fragment to the E. coli/B. subtilis plasmid pHV33 (available from the American Type Culture Collection as A.T.C.C. 39217) [Primrose, et al. Plasmid, 6, 193-201 (1981)] obtained from R. Dedonder (Institut Pasteur, Paris, France). The pHV33 plasmid had been previously digested with
15   EcoRI and PstI so that the xylE-containing fragment, when ligated in this region, would inactivate a gene for ampicillin resistance. The resulting plasmid, pAMB21, contains a functional xylE gene in E. coli host cells, but requires the addition of a promoter for xylE to be expressed in B. subtilis host cells. E. coli cells harboring pAMB21 are resistant to tetracycline (15 μg/ml) and chloramphenicol (20 μg/ml) while B. subtilis cells harboring pAMB21 are resistant only to chloramphenicol (5 μg/ml).
20   The t_{oop} transcription termination sequence of bacteriophage lambda was transferred from plasmid pCFM936 (on a 400 base pair PstI to BglII fragment) to the unique PstI site of pAMB21. A synthetic nucleotide with the sequence, 5′ GATCTGCA 3′, was constructed to join the BglII extremity of the t_{oop} fragment to the PstI site of the vector pAMB21. The resulting plasmid was designated pAMB22 and had properties identical to pAMB21 except for the inclusion of a transcription terminator. The pAMB22 plasmid is useful for detecting strong promoters that are functional in B. subtilis.
25   The 1.4 kb EcoRI to BglII fragment of DNA from pAMB22 that contains xylE and t_{oop} was isolated from a low-melting point agarose gel after electrophoresis of restricted fragments. The 1.4 kb piece of DNA was ligated to plasmid pBD64 (available from Bacillus Genetic Stock Center, number 1E22) which had been previously digested with EcoRI and BamHI. The resulting 5.3 kb plasmid, pAMB11, contains the polylinker sequence of M13mp18 (EcoRI, SstI, XmaI, Sma, BamHI and XbaI) upstream of the xylE gene which is followed by t_{oop}, as shown in Figure 3. The pAMB11 plasmid
30   is capable of replicating in B. subtilis and confers upon host cells resistance to chloramphenicol (5 μg/ml) and/or kanamycin (5 μg/ml).

As illustrated in Fig. 4, the purified EcoRI to KpnI fragment containing aprA was cloned onto pAMB11 to form pAMB111. Ligation products were introduced into B. subtilis MI112 (arg-15 leuB thr5 recE4) (available from Bacillus Genetic Stock Center as No. 1A423) by the protoplast transformation method described by Chang, et al., Mol. Gen.
35   Genet., 168, 111-115 (1979). B. subtilis MI112 without plasmid DNA is protease-proficient (Prt+ phenotype), but secreted levels of subtilisin are rather low. Chloramphenicol-resistant (Cm^r) transformants were transferred onto L-agar plates supplemented with 1.5% (w/v) skim milk and 5 μg/ml chloramphenicol, then incubated at 37°C.

After incubation at 37°C for approximately sixteen hours, colonies of MI112 harboring plasmid pAMB111 produced a clear halo surrounding each colony. Halos were formed by the proteolytic action of subtilisin on the casein component
40   of the skim milk medium supplement. MI112 harboring the pAMB11 vector alone had no visible halo after 16 hrs. of incubation, although a slight halo eventually developed after 40 hrs. of incubation at 37°C. Cells carrying pAMB111 were clearly distinguished from cells carrying pAMB11 by a difference in halo size. The cloning of the aprA gene in a fully functional form thus led to a high level production and secretion of subtilisin by B. subtilis.

45   ## Example 2

As illustrated in Fig. 4, a 3.0 kb EcoRI to KpnI genomic fragment, the isolation of which is described in Example 1, was digested with HindIII to produce three fragments: (1) a 1.1 kb EcoRI to HindIII fragment carrying genetic regulatory sequences for aprA gene expression, the "pre-pro" region of the gene required to extracellular export of subtilisin,
50   and the DNA sequence coding for the first 49 amino acids of mature subtilisin; (2) a 1.1 kb HindIII to HindIII fragment carrying DNA sequences coding for amino acids 50 through 275 (carboxyl-terminus) of subtilisin along with a transcription termination sequence and 3′ non-coding sequences; and (3) a 0.8 kb HindIII to KpnI fragment containing 3′ non-coding sequences.

The 1.1 kb fragment flanked by HindIII sites was cloned to the single HindIII site of bacteriophage M13 mp18 for
55   the purposes of DNA sequencing and site-directed mutagenesis. One of the recombinants, designated M13 mp18 apr2, provided single stranded template DNA required for site-directed mutagenesis of the aprA gene.

The coding region of the aprA gene was sequenced and the results of the sequence are set forth in Table 1 herein. It should be noted that the specific identity of the initial 5 codons of the leader region is attributable to the report of

Stahl, et al., supra, and Wong, et al., supra, of sequence information for the aprA gene, and that there exist codon sequence differences from Stahl, et al., supra, at amino acid positions 84 and 85. Specifically, Stahl, et al., supra, reports a codon GTT (coding for valine) at amino acid position 84 while the codon GTA (also coding for valine) appears in Table 1. Stahl, et al., supra, also reports a codon AGC (coding for serine) at amino acid position 85 as opposed to the codon GCG (coding for alanine) in Table 1.

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TABLE 1

-105
```
Met Arg Ser Lys Lys Leu Trp Ile Ser Leu Leu Phe Ala
GTG AGA AGC AAA AAA TTG TGG ATC AGC TTG TTG TTT GCG


Leu Thr Leu Ile Phe Thr Met Ala Phe Ser Asn Met Ser Ala
TTA ACG TTA ATC TTT ACG ATG GCG TTC AGC AAC ATG TCT GCG


Gln Ala Ala Gly Lys Ser Ser Thr Glu Lys Lys Tyr Ile Val
CAG GCT GCC GGA AAA AGC AGT ACA GAA AAG AAA TAC ATT GTC


Gly Phe Lys Gln Thr Met Ser Ala Met Ser Ser Ala Lys Lys
GGA TTT AAA CAG ACA ATG AGT GCC ATG AGT TCC GCC AAG AAA


Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln
AAG GAT GTT ATT TCT GAA AAA GGC GGA AAG GTT CAA AAG CAA


Phe Lys Tyr Val Asn Ala Ala Ala Ala Thr Leu Asp Glu Lys
TTT AAG TAT GTT AAC GCG GCC GCA GCA ACA TTG GAT GAA AAA


Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tyr Val
GCT GTA AAA GAA TTG AAA AAA GAT CCG AGC GTT GCA TAT GTG

                                        -1  +1
Glu Glu Asp His Ile Ala His Glu Tyr Ala Gln Ser Val Pro
GAA GAA GAT CAT ATT GCA CAT GAA TAT GCG CAA TCT GTT CCT

                10
Tyr Gly Ile Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln
TAT GGC ATT TCT CAA ATT AAA GCG CCG GCT CTT CAC TCT CAA

20                                      30
Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser
GGC TAC ACA GGC TCT AAC GTA AAA GTA GCT GTT ATC GAC AGC

                        40
Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Arg Gly Gly
GGA ATT GAC TCT TCT CAT CCT GAC TTA AAC GTC AGA GGC GGA

            50                                      60
Ala Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp Gly
GCA AGC TTC GTA CCT TCT GAA ACA AAC CCA TAC CAG GAC GGC

                        70
Ser Ser His Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu
AGT TCT CAC GGT ACG CAT GTA GCC GGT ACG ATT GCC GCT CTT
```

9

TABLE 1 (cont'd.)

```
                         80
Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser
AAT AAC TCA ATC GGT GTT CTG GGC GTA GCG CCA AGC GCA TCA

   90                                      100
Leu Tyr Ala Val Lys Val Leu Asp Ser Thr Gly Ser Gly Gln
TTA TAT GCA GTA AAA GTG CTT GAT TCA ACA GGA AGC GGC CAA

                             110
Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ser Asn
TAT AGC TGG ATT ATT AAC GGC ATT GAG TGG GCC ATT TCC AAC

       120                                        130
Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Thr Gly
AAT ATG GAT GTT ATC AAC ATG AGC CTT GGC GGA CCT ACT GGT

                             140
Ser Thr Ala Leu Lys Thr Val Val Asp Lys Ala Val Ser Ser
TCT ACA GCG CTG AAA ACA GTC GTT GAC AAA GCC GTT TCC AGC

           150
Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser
GGT ATC GTC GTT GCT GCC GCA GCC GGA AAC GAA GGT TCA TCC

160                                    170
Gly Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser
GGA AGC ACA AGC ACA GTC GGC TAC CCT GCA AAA TAT CCT TCT

                         180
Thr Ile Ala Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala
ACT ATT GCA GTA GGT GCG GTA AAC AGC AGC AAC CAA AGA GCT

       190                                      200
Ser Phe Ser Ser Ala Gly Ser Glu Leu Asp Val Met Ala Pro
TCA TTC TCC AGC GCA GGT TCT GAG CTT GAT GTG ATG GCT CCT

                         210
Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Gly Thr Tyr Gly
GGC GTG TCC ATC CAA AGC ACA CTT CCT GGA GGC ACT TAC GGC

               220
Ala Tyr Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly
GCT TAT AAC GGA ACG TCC ATG GCG ACT CCT CAC GTT GCC GGA

230                                    240
Ala Ala Ala Leu Ile Leu Ser Lys His Pro Thr Trp Thr Asn
GCA GCA GCG TTA ATT CTT TCT AAG CAC CCG ACT TGG ACA AAC

                   250
Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr Leu
GCG CAA GTC CGT GAT CGT TTA GAA AGC ACT GCA ACA TAT CTT
```

TABLE 1 (cont'd.)

```
                260                                                    270
   Gly Asn Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln
   GGA AAC TCT TTC TAC TAT GGA AAA GGG TTA ATC AAC GTA CAA


                275
   Ala Ala Ala Gln OC
   GCA GCT GCA CAA TAA TAGTAAAAAGAAGCAGGTTCCTCCATACCTGCT


   TCTTTTTATTTGTCAGCATCCTGATGTTCCGGCGCATTCTC
```

Bacteriophage M13 mp18 apr2 was constructed by inserting a 1.1 kb HindIII to HindIII fragment of B. subtilis QB127 genomic DNA, carrying nucleotide sequences coding for amino acids 50 through 275 (carboxyl-terminus) of aprA - subtilisin along with a transcription termination sequence and 3' non-coding sequences, in the unique HindIII site of bacteriophage M13 mp18. To eliminate the 3' non-coding sequences, a KpnI restriction endonuclease site was introduced, by site-directed mutagenesis, at a position immediately following the transcription termination sequence.

Site-directed mutagenesis was conducted in accordance with a procedure described by Norrander et. al., Gene, 26, 101-106 (1983). Single-stranded DNA from M13 mp18 apr2 was annealed to a primer,

```
                    *    *
        5'  TCCTGAGGTACCGGCGCATTC  3'
```

which was synthesized by the phosphite method described by Beaucage et. al., Tetrahedron Letters 22, 1859-1862 (1981). The primer was homologous to the nucleotides in this region except for two (marked by asterisks), where a thymine (T) was changed to guanine (G) and another thymine (T) was changed to adenine (A), thus creating a KpnI site (underlined) in this region.

The primer was annealed to M13 mp18 apr2 DNA at 65°C and the annealed DNA was slowly cooled to approximately 22°C and then polymerized for 2 hr. at 15°C in a reaction mixture which consisted of 12.5 μl of annealed DNA solution, 2.5 μl of 10 mM each of dATP, dCTP and dGTP, 20 μl of 12 mM ATP, 0.1 μl Klenow DNA polymerase, 0.1 μl T4 DNA ligase and 13 μl sterile distilled water. The resulting double-stranded, covalently closed circular DNA was introduced into E. coli JM103 by transfection.

Bacteriophage plaques were then transferred to Gene Screen ™(New England Nuclear, Beverly, Massachusetts) hybridization membranes. Plaques which contained DNA with the desired base changes were identified by hybridization to the radioactively labeled (γ-³²P) synthetic oligonucleotide used for the mutagenic priming reaction described above. Hybridization was performed at a restrictive temperature (65°C) in order that only DNA carrying a KpnI mutation would hybridize to the synthetic oligonucleotide. The presence of the KpnI mutation downstream of the aprA gene on DNA from a single purified plaque, designated M13 mp18 apr2 KpnI, was confirmed by DNA sequencing by the procedure described by Sanger et. al., supra and restriction enzyme analysis.

A 1.1 kb segment carrying most of the 3' non-coding region was deleted by digesting M13 mp18 apr2 KpnI with KpnI, religating digestion products at a concentration of 500 ng DNA/ml, then introducing the ligation products into E. coli JM103 by transfection. Bacteriophage plaques which contained DNA with the desired 0.35 kb deletion were identified by restriction endonuclease analysis. Bacteriophage from one such plaque was designated M13 mp18 apr4 (Fig. 7). M13 mp18 apr4 provided single-stranded template DNA for site-directed mutagenesis of the aprA gene described hereinafter.

Example 3

In order to express mutated subtilisin genes in B. subtilis, the plasmid pAMB106 was constructed as a vehicle for the mutated gene, as follows:

1) pAMB111 was digested with HindIII. A 1.1 kb segment carrying most of the aprA gene was deleted by re-ligating HindIII digestion products of pAMB111 at a concentration of approximately 1 μg/ml. This resulted in the formation of pAMB110 as illustrated in Fig. 4. The pAMB110 plasmid carries genetic regulatory sequences for expression of the subtilisin gene, the "pre-pro" region required for secretion of subtilisin, and the DNA sequence coding for the 3' non-coding region of mature subtilisin and the first 49 amino acids of mature subtilisin.

2) Plasmid pAMB110 was digested with BamHI and PstI in combination. This produced DNA fragments of two sizes, 6.2 kb and 1.0 kb. The 1.0 kb fragment carries the xylE gene, coding for catechol 2,3-dioxygenase, from the TOL plasmid of Pseudomonas putida mt-2 (Zukowski et. al., supra).

3) The larger, 6.2 kb BamHI-PstI fragment was self-ligated with the aid of a single-stranded synthetic oligonucleotide, 5′ GATCTGCA 3′, which was synthesized by the phosphite method described by Beaucage et. al., supra, and T4 DNA ligase. Ligation products were introduced into B. subtilis MI112 (arg-15 leuB thr5 recE4 (available from Bacillus Genetic Stock Center as No. 1A423) by the protoplast transformation method described by Change et. al., Mol. Gen. Genet. 168, 111-115 (1979).

Chloramphenicol-resistant (CmR) colonies were screened for plasmid content. The 6.2 kb plasmid pAMB106 was identified by restriction endonuclease analysis. It is identical to plasmid pAMB110 except that xylE has been deleted (Figure 6).

Because it is lacking DNA coding for amino acids 50 through 275 of aprA subtilisin, pAMB106 does not synthesize subtilisin when introduced into B. subtilis host cells. Subtilisin is synthesized only after insertion of the remainder of the subtilisin gene, i.e., either the native DNA sequence or an analog-encoding sequence.

Example 4

Preparation of a [Serine 109] Subtilisin Analog

Single-stranded DNA from bacteriophage M13mp18 apr4 was annealed to a primer,

$$\overset{*}{}$$

```
5'  TGG ATT ATT AGC GGC ATT GAG TGG  3'
    106 107 108 109 110 111 112 113
    TRP ILE ILE SER GLY ILE GLU TRP
```

which was synthesized by the phosphite method described by Beaucage et. al., supra. The primer was homologous to the nucleotides comprising codons for amino acids 106 through 113 of aprA-subtilisin except for one base change (marked by an asterisk) where an A was changed to a G to allow for the transition which would change Asn^{109} (codon AAC) to Ser^{109} (codon AGC).

The primer was annealed to M13mp18 apr4 DNA at 65°C and the annealed DNA was slowly cooled to approximately 22°C and then polymerized, ligated and transfected as described in Example 2.

Bacteriophage plaques were transferred to hybridization membranes, then those which contained DNA with the desired base change were identified by hybridization to a radioactively labeled (α-^{32}P) oligonucleotide used for the mutagenic priming reaction described above. Hybridization was performed at 65°C. One positive plaque contained bacteriophage designated as M13mp18 apr4[Ser^{109}]. Double-stranded DNA from this bacteriophage was digested with HindIII and KpnI in combination, then the 750 bp fragment carrying the mutated portion of the aprA-subtilisin gene was ligated to pAMB106 which had been previously digested with HindIII and KpnI. The resulting plasmid, pAMB129, may be introduced into a suitable B. subtilis host cells for synthesis and secretion of [Ser^{109}]-subtilisin.

Example 5

Preparation of a [Serine^{109}, Serine ^{218}] Subtilisin Analog

Single-stranded DNA from M13mp18 apr4[Ser^{109}] was annealed to a primer:

$$\overset{*}{}$$

```
5'  GGC GCT TAT AGC GGA AC  3'
    215 216 217 218 219 220
    GLY ALA TYR SER GLY THR
```

which was synthesized by the phosphite method described by Beuacage et. al., supra. The primer was homologous to nucleotides comprising codons for amino acids 215 through 220 of aprA-subtilisin except for one base change (marked by an asterisk) where an A was changed to a G to allow for the transition which would change Asn^{218} (codon AAC) to Ser^{218} (codon AGC). The conditions for annealing, polymerization, ligation, transfection, and identification of positive plaques were as described in Example 2. A single purified plaque contained bacteriophage designed as M13mp18 apr4 [Ser^{109}, Ser^{218}]. Double-stranded DNA from this bacteriophage was digested with HindIII and KpnI in

combination, then a 750 bp fragment carrying the two mutations was ligated to pAMB106 which had been previously digested with HindIII and KpnI. The resulting plasmid, pAMB130, may be introduced into B. subtilis host cells for synthesis and secretion of [Ser[109], Ser[218]]-subtilisin.

Example 6

Preparation of a [Asp[76], Ser[109], Ser[218]] Subtilisin Analog

Single-stranded DNA from M13mp18 apr4 [Ser[109], Ser[218]] was annealed to a primer:

$$\overset{*}{}$$

5' GCT CTT GAT AAC TCA ATC 3'

  74   75   76   77   78   79

ALA LEU ASP ASN SER ILE

which was synthesized by the phosphite method described by Beaucage et. al., supra. The primer was homologous to the nucleotides comprising codons for amino acids 74 through 79 of aprA-subtilisin except for one base change (marked by an asterisk), where an A was changed to a G to allow for the transition which would change Asn[76] (codon AAT) to Asp[76] (codon GAT).

The primer was annealed to M13mp18 [Ser[109], Ser[218]] DNA at 65°C and the annealed DNA was slowly cooled to approximately 22°C and polymerized, ligated and transfected as described in Example 2.

Bacteriophage plaques were transferred to hybridization membranes and those which contained DNA with the desired base change were identified by hybridization as described in Example 2 except that hybridization was performed at 46°C. One positive plaque contained bacteriophage designated at M13mp18 apr4 [Asp[76], Ser[109], Ser[218]]. Double-stranded DNA from the bacteriophage was digested with HindIII and KpnI in combination, then a 750 bp fragment carrying the three mutations of the aprA-subtilisin gene was ligated to pAMB106 which had been previously digested with HindIII and KpnI. The resulting plasmid, pAMB131, may be introduced into B. subtilis host cells for synthesis and secretion of [Asp[76], Ser[109], Ser[218]]-subtilisin.

Example 7

Preparation of a [Asp[76], Glu[79], Ser[109], Ser[218]] Subtilisin Analog

Single-stranded DNA from M13mp18 apr4 [Asp[76], Ser[109], Ser[218]] was annealed to a primer:

$$* * *$$

5' T GAT AAC TCA GAA GGT GTT CTG G   3'

  75 76   77   78   79   80   81   82   83

ASP ASN SER GLU GLY VAL LEU

which was synthesized by the phosphite method described by Beaucage et al., supra. The primer was homologous to the nucleotides comprising partial codons for amino acids 75 and 83 and entire codons for amino acids 76 through 75 and 83 and entire codons for amino acids 76 through 82 of [Asp[76], Ser[109], Ser[218]]-subtilisin except for three base changes (marked by asterisks), wherein an A was changed to a G, a T was changed to an A, and a C was changed to an A, which changed Ile[79] (codon ATC) to Glu[79] (codon GAA).

The primer was annealed to M13mp18 apr4 [Asp[76], Ser[109], Ser[218]] DNA at 65° and the annealed DNA was slowly cooled to approximately 22°C and was polymerized, ligated and transfected as described in Example 2.

Bacteriophage plaques were transferred to hybridization membranes and those which contained the desired base changes were identified by hybridization as described in Example 2 except that hybridization was performed at 45°C. One positive plaque contained bacteriophage designated as M13mp18 apr4 [Asp[76], Glu[79], Ser[109], Ser[218]]. Double-stranded DNA from this bacteriophage was digested with HindIII and KpnI in combination, then a 750 bp fragment carrying the four mutations of the aprA-subtilisin gene was ligated to pAMB106 which had ben previously digested with HindIII and KpnI. The resulting plasmid, pAMB133, may be introduced into B. subtilis host cells for synthesis and secretion of [Asp[76], Glu[79], Ser[109], Ser[218]]-subtilisin.

Example 8

Preparation of a [Asp[76], Ser[109], Ser[218], Ala[222]]-Subtilisin Analog

Single-stranded DNA from M13 mp18 apr4 [Asp[76], Ser[109], Ser[218]] was annealed to the primer:

$$
\begin{array}{ccccccc}
 & & \ast & \ast & & \\
5' & GGA & ACG & TCC & GCG & GCG & ACT & 3' \\
 & 219 & 220 & 221 & 222 & 223 & 224 & \\
 & Gly & Thr & Ser & Ala & Ala & Thr &
\end{array}
$$

which was synthesized by the phosphite method described by Beaucage et al., supra. The primer was homologous to the nucleotides comprising codons for amino acids 219 through 224 of [Asp[76], Ser[109], Ser[218]]-subtilisin except for two base changes (marked by asterisks), wherein an adenine (A) was changed to a guanine (G) and a thymine (T) was changed to a cytosine (C), which changed the Met[222] (codon ATG) to Ala[222] (codon GCG). The primer was annealed to M13mp18 apr4 [Asp[76], Ser[109], Ser[218]] DNA at 65°C and the annealed DNA was slowly cooled to approximately 22°C and was polymerized, ligated and transfected as described in Example 2.

Bacteriophage plaques were transferred to hybridization membranes and those which contained desired base changes were identified by hybridization as described in Example 2 except that hybridization was performed at 58°C. One positive plaque contained bacteriophage designated as M13 mp18 apr4 [Asp[76], Ser[109], Ser[218], Ala[222]]. Double stranded DNA from this bacteriophage was digested with HindIII and KpnI in combination, then at 750 bp fragment of DNA carrying the four mutations of the aprA-subtilisin gene was ligated to pAMB106 which had been previously digested with HindIII and KpnI. The resulting plasmid, pAMB143, may be introduced and propagated in Bacillus host cells for synthesis and secretion of [Asp[76], Ser[109], Ser[218], Ala[222]]-subtilsin.

Example 9

Preparation of M13 mp19 aprA143

Plasmid pAMB143 was digested with EcoRI and KpnI in combination. A 1.8 kb fragment of DNA carrying the entire gene for [Asp[76], Ser[109], Ser[218], Ala[222]]-subtilisin, along with its flanking sequences required for initiation of transcription and translation and termination of transcription and translation, was transferred to bacteriophage M13mp19 (available from Bethesda Research Laboratories, Gaithersburg, MD., as catalogue no. 8229SA) which had been digested with EcoRI and KpnI. One of the recombinant bacteriophage DNA resulting from this procedure was designated M13mp19 aprA143, and provided single-stranded template DNA required for site-directed mutagenesis of the [Asp[76], Ser[109], Ser[218], Ala[222]]-subtilisin gene.

Example 10

Preparation of a [Leu[31], Asp[76], Ser[109], Ser[218], Ala[222]]- Subtilisin Analog

Single-stranded DNA from M13 mp19 aprA143 was annealed to the primer:

$$
\begin{array}{ccccccc}
 & & & \ast & \ast & & \\
5' & GTA & GCT & GTT & TTA & GAC & AGC & GGA & 3' \\
 & 28 & 29 & 30 & 31 & 32 & 33 & 34 & \\
 & Val & Ala & Val & Leu & Asp & Ser & Gly &
\end{array}
$$

which was synthesized by the phosphite method described by Beaucage et al., supra. The primer was homologous to the nucleotides comprising codons for amino acids 28 through 34 of [Asp[76], Ser[109], Ser[218], Ala[222]]-subtilisin except for two base changes (marked by asterisks), wherein an adenine (A) was changed to thymine (T) and a cytosine (C) was changed to adenine (A), which changed the Ile[31] (codon ATC) to Leu[31] (codon TTA). The primer was annealed to M13 mp19 aprA143 DNA at 65°C and the annealed DNA was slowly cooled to approximately 22°C and was polymerized, ligated and transfected as described in Example 2.

Bacteriophage plaques were transferred to hybridization membranes and those which contained desired base changes were identified by hybridization as described in Example 2 except that hybridization was performed at 57°C.

One positive plaque contained bacteriophage designated as M13mp19, aprA144 and carried DNA coding for [Leu[31], Asp[76], Ser[109], Ser[216], Ala-2]-subtilisin. Double-stranded DNA from this bacteriophage was digested with EcoRI and KpnI in combination, then a 1.8 kb fragment of DNA carrying the five mutations of the aprA-subtilisin gene was ligated to pAMB106 which had been previously digested with EcoRI and KpnI. The resulting plasmid, pAMB144, may be introduced and propagated in Bacillus host cells for synthesis and secretion of [Leu[31], Asp[76], Ser[109], Ser[218], Ala[222]]-subtilisin.

Example 11

Preparation of a [Asp[76], Ser[109], Leu[124], Ser[218], Ala[222]]-Subtilisin Analog.

Single-stranded DNA from M13mp19 aprA143 was annealed to the primer:

$$\text{5' AT GTT ATC AAC TTA AGC CTT GG 3'}$$

```
              *    *
5'   AT GTT ATC AAC TTA AGC CTT GG 3'
        121 122 123 124 125 126
        Val Ile Asn Leu Ser Leu
```

which was synthesized by the phosphite method described by Beaucage et al., supra. The primer was homologous to the nucleotides comprising partial codons for amino acids 120 and 127 and complete codons for amino acids 121 through 126 of [Asp[76], Ser[109], Ser[218], Ala[222]]-subtilisin except for two base changes (marked by asterisks), wherein an adenine (A) was changed to thymine (T) and a guanine (G) was changed to adenine (A), which changed the Met[124] (codon ATG) to Leu[124] (codon TTA).

The primer was annealed to M13mp19 aprA143 DNA at 65°C and the annealed DNA was slowly cooled to approximately 22°C and was polymerized, ligated and transfected as described in Example 2.

Bacteriophage plaques were transferred to hybridization membranes and those which contained desired base changes were identified by hybridization as described in Example 2 except that hybridization was performed at 59°C. One positive plaque contained bacteriophage designated as M13 mp19 aprA145 and carried DNA coding for [Asp[76], Ser[109], Leu[124], Ser[218], Ala[222]]-subtilisin. Double-stranded DNA from this bacteriophage was digested with EcoRI and KpnI in combination, then a 1.8 kb fragment of DNA carrying the five mutations of the aprA-subtilisin gene was ligated to pAMB106 which had been previously digested with EcoRI and KpnI. The resulting plasmid, pAMB145, may be introduced and propagated in Bacillus host cells for synthesis and secretion of [Asp[76], Ser[109], Leu[124], Ser[218], Ala[222]]-subtilisin.

Example 12

Because most Bacilli secrete alkaline and/or neutral proteases into the surrounding growth medium, it is preferable that mutations be introduced into endogenous alkaline and neutral protease genes of B. subtilis to block their synthesis so that mutated subtilisin genes, when introduced into the mutant cell, may produce mutated subtilisins which will then be secreted in a medium free of other proteases likely to interfere with isolation of intact subtilisin analogs. Two mutant B. subtilis strains BZ24 and BZ25, which produce no detectable extracellular proteases, were constructed in accordance with the following procedure:

First, a plasmid vehicle capable of replicating in E. coli, but not in B. subtilis unless integrated into the B. subtilis chromosome by homologous recombination, was constructed as follows. Plasmid pBD64 (Bacillus Genetic Stock Center, Number 1E22) was digested to completion with HpaII to produce three fragments of 2.95 kb, 1.0 kb and 0.75 kb in size. These fragments were then ligated as a mixture to plasmid pBR322 (A.T.C.C. 37017) which previously had been digested with ClaI. The ligation products were introduced into E. coli C600 (available from the American Type Culture Collection as A.T.C.C. 23724) by transformation [Mandel, et al., J. Mol. Biol., 53, 154 (1970)]. Selection was for cells resistant to chloramphenicol (20 μg/ml) and ampicillin (50 μg/ml). Plasmid DNA from 12 transformants was prepared by an alkaline extraction procedure described by Birnboim, et al., Nucleic Acids Res., 7, 1513-1523 (1979), then digested with HindIII and EcoRI in combination to verify the presence of inserted fragment(s). One such plasmid, designated pAMB30, was found to carry the 1.0 and 0.75 kb HpaII fragments of pBD64 in the ClaI site of pBR322. These fragments contain the chloramphenicol acetyltransferase (cat) gene which is functional in E. coli and B. subtilis. Digestions with BglII and, separately, with Sau3A confirmed the identity and orientation of the cat gene on pAMB30, as illustrated in Fig. 5.

Because pAMB30 lacks an origin of replication sequence which is functional in B. subtilis, it cannot replicate as

an autonomous replicon in B. subtilis host cells. On the other hand, pAMB30 contains the pBR322-derived origin of replication which is functional in E. coli, thus the plasmid can be propagated in E. coli host cells. Plasmid pAMB30 is useful in at least 2 ways. First, a fragment of DNA which contains a functional origin of replication in B. subtilis may be detected when cloned onto pAMB30 such that the plasmid will autonomously replicate in the extrachromosomal state.

5 Second, plasmid pAMB30 can integrate into the genome of B. subtilis at a site of homology between the chromosome and B. subtilis DNA cloned onto pAMB30. This has been demonstrated by Haldenwang, et al., J. Bacteriol., 142, 90-98 (1980) and Young, J. Gen. Microbiol., 129, 1497-1512 (1983) using plasmid vehicles similar to, but not identical to pAMB30.

Plasmid pAMB21 (described in Example 1) was digested with EcoRI and PstI to isolate the xylE gene on a 1.0 kb

10 fragment. The fragment was ligated to pAMB30 which had been previously digested with EcoRI and PstI. Ligation products were introduced into E. coli C600 by transformation. Selection was for chloramphenicol resistant (20 µg/ml) host cells which were sensitive to ampicillin (50 µg/ml) due to the insertion of the xylE fragment of pAMB21 into the structural gene for ampicillin resistance of pAMB30. The resulting plasmid, pAMB30/21, has properties identical to pAMB30 but has, in addition, a functional xylE gene.

15 Plasmid pAMB110, which carries the aprA gene deleted of a region coding for the latter 226 amino acids of mature subtilisin, was digested with EcoRI and KpnI. The 1.9 kb fragment of B. subtilis DNA containing genetic regulatory sequences for aprA gene expression, "the pre-pro" region, the DNA sequence coding for the first 49 amino acids of mature subtilisin and 3′ non-coding sequences was ligated to pAMB30/21 that had been previously digested with EcoRI and KpnI. Ligation products were introduced into E. coli C600 by transformation. Plasmid DNA from several transform-

20 ants was isolated by the alkaline extraction procedure of Birnboim, et al., supra, and the presence of the inserted 1.9 kb fragment was verified by multiple restriction endonuclease digestions. One such plasmid, designated pAMB301, was retained for further use.

B. subtilis strain BGSC1A274 (Bacillus Genetic Stock Center) carries a mutation at the npr locus and is incapable of producing extracellular neutral protease. The plasmid pAMB301 was integrated into the genome of B. subtilis

25 BGSC1A274 by transformation of competent cells [Spizizen, Proc. Natl. Acad. Sci. (USA), 44, 1072-1078 (1958)]. Selection was for chloramphenicol-resistant (5 µg/ml) host cells which were then transferred by sterile toothpicks to L-agar supplemented with 1.5% (w/v) powdered skim milk and (5 µg/ml) cloramphenicol. Those cells which failed to produce a clear halo surrounding the colony were deficient in the ability to produce extracellular neutral and serine proteases due to the combination of the npr mutation along with the newly introduced aprA mutation. The aprA mutation

30 was a deletion of the latter 226 amino acids of mature subtilisin due to the replacement of the wild-type aprA gene with the deleted version carried on pAMB301. One such strain, designated BZ24, has the Npr⁻ Apr⁻ Cmʳ phenotype, thus it produces no detectable extracellular neutral protease nor extracellular alkaline protease and is resistant to chloramphenicol at 5 µg/ml. Southern blotting [Southern, J. Mol. Biol., 98, 503-517 (1975)] was used to confirm the deletion in the aprA gene on the chromosome of B. subtilis BZ24. Cultivation of B. subtilis BZ24 in Antibiotic Medium No. 3 (Pe-

35 nassay Broth, Difco, Detroit, Michigan) in the absence of antibiotic selection for approximately 32 generations led to the isolation of a derivative strain of BZ24 in which the cat gene confering chloramphenicol resistance upon host cells was lost due to its instability in the BZ24 chromosome. Such a phenomenon has been previously observed by Stahl, et al., J. Bacteriol., 158, 411-418 (1984). A chloramphenicol-sensitive derivative of B224 was designated BZ25. B. subtilis BZ25 has the Npr⁻ Apr⁻ phenotype, thus it produces no detectable extracellular neutral protease nor extracellular

40 alkaline protease. Southern blotting was used to confirm the deletion in the aprA gene on the chromosome of B. subtilis BZ25.

Because B. subtilis BZ25 produces no detectable extracellular neutral protease nor subtilisin, it is a useful host strain for introduction of plasmid DNA, such as pAMB113, for the production of mutated subtilisins which may be secreted into the surrounding growth medium free of other proteases.

45 B. subtilis BZ25 produces no detectable extracellular proteases when culture supernatants are assayed as described below. B. subtilis BZ25/pAMB113, which is BZ25 that harbors plasmid pAMB113 (introduced by the protoplast transformation method of Chang, et al., supra) produces appreciable quantities of [Ser²¹⁸]-subtilisin when culture supernatants are assayed as described.

50 Example 13

Integration of the [Ser²¹⁸]-subtilisin gene into the chromosome of B. subtilis was believed to provide an efficient way of increasing the genetic stability of this mutant gene. Such an approach also alleviates the requirement for chloramphenicol in the fermentation medium which is otherwise needed for application of selective pressure to maintain

55 plasmid DNA in the extrachromosomal state. Therefore, the [Ser²¹⁸]-subtilisin gene, along with its genetic regulatory sequences and flanking DNA homologous to the B. subtilis chromosome, was isolated from a low melting point agarose gel after electrophoresis of pAMB113 which had been digested with EcoRI and PstI in combination. The 4.0 kb EcoRI to PstI fragment (illustrated in Fig. 4) was then ligated to pAMB30 (illustrated in Fig. 5) which had been digested with

EcoRl and PstI in combination. Ligation products were introduced into E. coli HB101 (A.T.C.C. 33694) by transforma-
tion. Selection was for cells resistant to chloramphenicol (20 */µg/ml). Plasmid DNA from four transformants which met
the criteria above were isolated by the alkaline extraction procedure of Birnboim, et al., supra, then digested with EcoRl
and PstI in combination. All four plasmids contained the 4.0 kb insert and the 5.6 kb remaining portion of pAMB30.
One such plasmid, designated pAMB302, was purified and retained for further use.

Repeated attempts to integrate plasmid pAMB302 into the chromosome of B. subtilis BZ25 by the competence
method [Spizizen, supra] were unsuccessful. This may have been due to the failure of BZ25 cells to become competent
by the method employed. Therefore, pAMB302 was introduced into B. subtilis BZ25 cells by the protoplast transfor-
mation method of Chang, et al., supra. This result is particularly significant in that research strains in which integration
has been obtained were selected on the basis of transformation by the competence method. Strains which may be
unable to become competent, and in particular industrial strains which were not selected on the basis of transformation
by the competence method, may be more likely to be unable to become competent.

Selection was for chloramphenicol-resistant cells (5 µg/ml) cells, which were then transferred with sterile toothpicks
to L-agar supplemented with 1.5% (w/v) skim milk and 5 µg/ml chloramphenicol. Cells were incubated overnight at
37°C. Clear halos of different diameters were observed around the Cmʳ colonies. This indicates that subtilisin was
produced and secreted by these cells. An attempt was made to isolate plasmid DNA from eight of these colonies by
the alkaline extraction method. No plasmid DNA was detected on agarose gels which were stained with ethidium
bromide (1 µg/ml) to visualize DNA after electrophoresis. The absence of extrachromosomal plasmid DNA in the Cmʳ
cells which produced subtilisin was a strong indication that pAMB302 had been integrated into the chromosome of B.
subtilis.

Several colonies resulting from this experiment were isolated and designated BZ28, BZ29, BZ30, BZ31, BZ32 and
BZ33. Each strain was grown overnight at 37°C with vigorous shaking in brain heart infusion medium (BHI, Difco)
supplemented with 5 µg/ml chloramphenicol. Culture supernatants were assayed for subtilisin activity. B. subtilis strains
BZ28, BZ29, BZ30, BZ31, BZ32 and BZ33 all produced subtilisin and secreted it into the surrounding growth medium,
some strains producing more than others. The amount of subtilisin observed in the liquid culture broth was directly
proportional to the size of the halo observed on skim milk L-agar plates. Because of the amounts of subtilisin secreted
by these cells differed, multiple copies of pAMB302 were integrated into the chromosome or gene amplification [Young,
J. Gen. Microbiol., 129, 1497-1512 (1983); Albertini, et al., J. Bacteriol., 162, 1203-1211 (1985)] had taken place.

Example 14

Wild-type subtilisin and subtilisin analogs were isolated and purified as follows. Each culture broth was centrifuged
at 15,000g for 30 minutes and protein in the clear supernatant was precipitated with $(NH_4)_2SO_4$ (600 g per liter). The
precipitate was collected by centrifugation, dissolved in 20 mM 2 [N-morpholino] ethanesulfonic acid (MES) pH 6.4.
The solution was made 30% in acetone, and the 30% acetone supernatant was collected by centrifugation. The su-
pernatant was then made 75% in acetone, and the 30-75% acetone pellet was filtered and dried under vacuum.

In order to further purify the enzyme, the dried precipitate was dissolved in water and the solution was dialyzed
against 5 mM MES buffer at pH 6.4. The dialyzed solution was passed through a column (2.5 x. 15cm) of S-Sepharose
FF at a rate of 2 ml per minute. After washing the column with 0.02M MES, the enzyme was eluted with a linear gradient
of NaCl in the same buffer, to 0.5M NaCl. Peak fractions were pooled and protein from the fractions containing the
enzyme, as identified by a color change in a sample of the fraction mixed with azocasein were dialyzed at 4°C against
5 mM MES pH 6.3 and then lyophilized.

Example 15

Pure subtilisin or subtilisin analog was applied to a Pharmacia FPLC Superose 12 column, and the material eluting
as the intact (not cleaved) protein was pooled, in 20 mM MES, 0.1 M NaCl, pH 6.4. Samples of wild type subtilisin, or
subtilisin analog of the present invention to be evaluated were incubated for 10 min. in the same buffer, the buffer plus
3% SDS, or 20 mM MES, 0.1 M NaCl, 5 mM $CaCl_2$ and 15 mM EDTA at the indicated temperature. The samples were
cooled to room temperature for 5 min. and then assayed for 20 min. at room temperature (20°C) in Tris-HCl, pH 8.0
with 0.6% azocasein to determine proteolytic activity. The proteolytic activity of each sample is expressed as a per-
centage of the original activity of either wild type or analog, at 20°C in 10 mM $CaCl_2$, and is represented in Table 2.

TABLE 2

Proteolytic Activity of Wild Type Subtilisin			
Temperature	0% SDS	3% SDS	0% SDS +15 mM EDTA
20	100	8	100
35	100	0	62
50	95	0	37
70	14	0	14
100	0	0	0
Activity of [Asp^{76}, Ser^{109}, Ser^{218}] Subtilisin Analog of Example 6			
Temperature	0% SDS	3% SDS	0% SDS +15 mM EDTA
20	100	55	91
50	100	12	94
100	5	0	5

Example 16

Intact subtilisins were obtained by FPLC on the Superose 12 column. The intact subtilisins were incubated for 30 minutes at room temperature (20°C) in 15 mM MES, 0.05 M NaCl, pH 6.3 containing either 4 mM CaCl$_2$ or 4 mM EDTA, and a varied amount of SDS. The proteolytic activity of the enzyme was then determined by a 20 min. incubation in 0.6% azocasein in Tris-Cl, pH8.0. The proteolytic activity of each sample evaluated is expressed in Table 3 as a percentage of the original activity of the sample in 0% SDS and 10 mM Ca^{2+}.

TABLE 3

Proteolytic Activity of Wild Type Subtilisin		
% SDS	4 mM Ca^{2+}	4 mM EDTA
0	100	94
0.1	100	76
0.25	100	45
0.50	76	13
0.75	63	3
1.0	60	0
2.0	29	0
3.0	17	0
Proteolytic Activity of [Asp^{76}, Ser^{109}, Ser^{218}] Subtilisin Analog		
% SDS	4 mM Ca^{2+}	4 mM EDTA
0	100	95
0.1	100	95
0.25	100	86
0.50	100	81
0.75	96	79
1.0	96	78
2.0	86	69
3.0	71	65

Example 17

For this experiment only the subtilisins were purified as described below.

Each culture broth was centrifuged at 15,000g for 30 minutes and protein in the clear supernatant was precipitated with (NH$_4$)$_2$SO$_4$ (600 g per liter). The precipitate was collected by centrifugation, triturated with 75% acetone, filtered

and dried under vacuum.

In order to further purify the enzyme, the dried precipitate was dissolved in water and the solution was filtered and then dialyzed against 0.02M sodium phosphate buffer at pH 6.3. The dialyzed solution was passed through a column (2.5 x. 15cm) of carboxymethyl cellulose at a rate of 2 ml per minute. After washing the column with 0.02M sodium phosphate (pH 6.3), the enzyme was eluted with the same buffer containing 0.15M NaCl. Peak fractions were pooled and protein from the fractions containing the enzyme, as identified by a color change in a sample of the fraction mixed with succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-p-nitroanilide (Vega Biochemicals), were precipitated by addition of 2.5 volumes of acetone. The precipitate was collected by centrifugation and then dissolved in 0.005M calcium acetate (about 1 ml per 10 mg). The resulting solution was dialyzed at 4°C against water and then lyophilized.

The stabilities of [Asp^{76}, Ser^{109}, Ser^{218}] subtilisin analog, [Asp^{76}, Glu^{79}, Ser^{109}, Ser^{218}] subtilisin analog and subtilisin Carlsberg were evaluated at three temperatures (25°C, 37°C and 50°C) in two buffer solutions (0.06M sodium phosphate, pH 9.0 or 0.12 M sodium glycinate, pH 11.0). The results are expressed in Table 4 as half-life of the enzymes under the specified conditions.

TABLE 4

A. In 0.12M sodium glycinate pH 11.0 + 0.2% SDS.			
Subtilisin	$t_{1/2}$ (25°C)	$t_{1/2}$(37°C)	$t_{1/2}$(50°C)
[Asp^{76}, Ser^{109}, Ser^{218}] analog	110 days	35.2 hrs	6.7 hrs
subtilisin Carlsberg	2 days	8.4 hrs	0.53 hr
[Asp^{76}, Glu^{79}, Ser^{109}, Ser^{218}] analog	154 days	35.3 hrs	7.8 hrs

B. In 0.06M sodium phosphate pH 9.0 + 0.2% SDS.			
Subtilisin	$t_{1/2}$ (25°C)	$t_{1/2}$(37°C)	$t_{1/2}$(50°C)
[Asp^{76}, Ser^{109}, Ser^{218}] analog	79.2 hrs	16.0 hrs	0.52 hr
subtilisin Carlsberg	17.3 hrs	2.4 hrs	0.18 hr
[Asp^{76}, Glu^{79}, Ser^{109}, Ser^{218}] analog	86.3 hrs	22.0 hrs	0.96 hr

C. In 0.12M sodium glycinate pH 11.0 + 5 mM EDTA.			
Subtilisin	$t_{1/2}$ (25°C)	$t_{1/2}$(37°C)	$t_{1/2}$(50°C)
[Asp^{76}, Ser^{109}, Ser^{218}] analog	28.7 hrs	1.87 hrs	0.25 hr
subtilisin Carlsberg	24 hrs	1.71 hrs	0.45 hr
[Asp^{76}, Glu^{79}, Ser^{109}, Ser^{218}] analog	21.5 hrs	1.42 hrs	0.20 hr

D. In 0.06M sodium phosphate pH 9.0 + 5 mM EDTA.			
Subtilisin	$t_{1/2}$ (25°C)	$t_{1/2}$(37°C)	$t_{1/2}$(50°C)
[Asp^{76}, Ser^{109}, Ser^{218}] analog	27.4 hrs	1.75 hrs	0.23 hr
subtilisin Carlsberg	26.3 hrs	1.68 hrs	0.32 hr
[Asp^{76}, Glu^{79}, Ser^{109}, Ser^{218}] analog	19.7 hrs	1.36 hrs	0.17 hr

EXAMPLE 18

Characterization of [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]-Subtilisin Analog.

[Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] - subtilisin was purified from B. subtilis BZ25/pAMB143 fermentation broth as described in Example 14. The thermal stability, stability in bleach, and specific activity of this subtilisin analog was examined as described below:

I. Thermal Stability.

The thermal stability of [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] - subtilisin and [Asp^{76}, Ser^{109}, Ser^{218}] - subtilisin and of the wild type enzyme (AprA) were determined using the thermal program of the Gilford Model Response II Spectro-photometer. Samples of purified protease in 20 mM MES, 0.1 M NaCl pH 6.3 were heated from 25°C to 95°C in the presence of various concentrations of Ca^{2+}. The temperature was increased at a rate of 0.5°C per minute, while following the decrease in absorbance at 287 nm. The melting temperature (Tm) is defined as the temperature where the

populations of the unfolded and folded states are equal; in other words, the temperature occurring midway in the transition from folded to unfolded protein. Heat denaturation is an irreversible reaction for subtilisin: in the absence of protease inhibitor the intact enzyme digests the unfolded form, in the presence of inhibitor the protein precipitates as it unfolds. Therefore, the Tm gives us a relative comparison of stability. The melting temperatures determined using this technique are given below.

TABLE 5

Thermal Stability			
Tm			
mMCa^{2+}	AprA	[Asp^{76}, Ser^{109}, Ser^{218}] Subtilisin	[Asp^{76}, Ser^{109} Ser^{218}, Ala^{222}]-Subtilisin
0	60	66	66
1	64	68	68
5	65	71	ND
10	66	72	71
30	68	73	73
ND = not determined			

The melting temperatures of the 2 analog subtilisins are very similar at all of the calcium concentrations examined, and are consistently higher than that of the AprA-subtilisin. This indicates that the substitution of Ala for Met at residue 222 did not affect negatively the increased stability and higher calcium binding affinity achieved with the substitutions at positions 76, 109 and 218.

II. Stability in Bleach.

As the purpose of removing the Met at position 222 was to decrease the susceptibility of the enzyme to oxidation, the stability of [Asp^{76}, Ser^{109}, Ser^{218}] subtilisin and [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]-subtilisin in bleach were compared. In the first experiment, the two analogs were incubated at 50°C, in 20 mM Tris 0.1 M NaCl pH 7.5, in the presence of 2% chlorox, 2% chlorox plus 1% SDS, or buffer alone. It should be noted that 2% chlorox bleach is a vast excess compared to the amount normally used by a consumer for laundering fabrics. Aliquots were removed at various times and the protease activity remaining was determined using the azocasein assay. The results are shown in Figure 8. The activity of both analogs is expressed as percent of activity in the presence of buffer alone, which remained constant throughout the course of the experiment. [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] retains more activity than [Asp^{76}, Ser^{109}, Ser^{218}] in the presence of 2% bleach, while both analogs appear to lose activity rapidly in the presence of 2% chlorox and 1% SDS, at 50°C. An interesting observation during this experiment was the fact that under identical conditions, using the same amounts of protein, [Asp^{76}, Ser^{109}, Ser^{218}] had a much higher activity than [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]. This will be discussed in more detail below.

The effect of bleach on the structure of [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] and [Asp^{76}, Ser^{109}, Ser^{218}] was also assessed using SDS-PAGE. Both analogs were incubated at 50°C in 20 mM Tris, 0.1 M NaCl, 1 mM PMSF, pH 7.5 in the presence of 2% chlorox, or 2% chlorox plus 1% SDS. At different times, 30 μl were removed, mixed with 15 μl of cold (4°C) sample buffer containing 1 mM PMSF and stored at 4°C until the experiment was finished. The samples were then subjected to SDS-PAGE on 12.5% polyacrylamide resolving gels. The protein was visualized by staining with Coomassie blue dye, and the amount of intact, folded protein (at the top of the gel), intact, unfolded protein (in the middle of the gel) and proteolytic products (even further down the gel) were quantified using laser densitometry. For analog [Asp^{76}, Ser^{109}, Ser^{218}], after 1 hr in 2% chlorox 45% of the protein remained properly folded, while 2 hr in bleach resulted in all of the protein unfolding, and much of it being digested. For analog [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] after 1 hr in bleach, 56% of the protease was still folded, and even following a 2 hr incubation in 2% chlorox at 50°C, 20% of the total protein remained intact. In the 2% bleach, 1% SDS solution the behavior of both analogs was very similar, with 75% still intact and folded after a 5 min incubation, decreasing to 27% of the total protein still folded at 15 min. [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] does not show greatly increased stability to the combination of denaturants (bleach and SDS) in the concentrations used for this particular assay, but does show enhanced resistance to bleach denaturation.

III. Activity

As mentioned earlier, analog [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] has a lower specific activity toward azocasein than analog [Asp^{76}, Ser^{109}, Ser^{218}], with an approximate Vm of 0.02 dA/(min-mg/ml) versus 0.08 dA/(min-mg/ml) for [Asp^{76},

Ser^{109}, Ser^{218}]. To see if this decreased activity was specific for azocasein, or a general characteristic of the [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] analog, the kinetics of the hydrolysis of the artificial peptide succinyl Ala-Ala-Pro-Phe-para-nitroanilide was analyzed for [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] and [Asp^{76}, Ser^{109}, Ser^{218}]. Analog [Asp^{76}, Ser^{109}, Ser^{218}] had a Km of 440 µM and a Vmax 7.9 dA/(min-nmole); both these values are consistent with those obtained in previous analyses. The Km of [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] for the substrate was 1.4 mM, while the Vmax was 1.1 dA/(min-nmole) This represents a three-fold decrease in substrate affinity coupled with a seven-fold decrease in specific activity. This specific activity is 4-fold less than that of aprA-subtilisin. The catalytic Ser is at position 221, and it appears that substitutions at position 222 interfere with substrate binding as well as with the rate limiting step of proteolysis.

EXAMPLE 19

As previously noted in Example 18, [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] - subtilisin has a lower specific activity toward azocasein than [Asp^{76}, Ser^{109}, Ser^{218}]-subtilisin and also has a lower specific activity toward the synthetic peptide sAAPFpN substrate. This result is consistent with the loss of activity in the Ala^{222} analog of BPN' subtilisin from Bacillus amyloliquefaciens as observed by Estell et al., J. Biol. Chem. 260, 6564-6570, 1986. Since casein or azocasein is commonly used as a substrate to determine activity of proteases by manufacturers of detergent enzymes and detergent formulators, the [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin was not expected to demonstrate improved performance in washing studies performed on soiled fabrics.

As illustrated in this Example, the [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]- subtilisin analog produced the unexpected result of consistently improved performance in removing stains from soiled fabrics under several conditions. The conditions in Figures 9 and 10 were as follows:

- Whole wash conditions, 10 minute wash
- Fresh Start™ with phosphate, pH approx 7 Fresh Start™ without phosphate, pH approx 9
- 80° and 120° F wash temperature
- 150 ppM water hardness
- 2 stains, 3 swatches each:
    Blood/Milk/Ink (BMI) on cotton
    Chocolate Fudge Pudding (CFP) on a 65% dacron/35% cotton fabric
- Controls: 1.5% Alcalse™ m Fresh Start™ 1.5% Termamyl™ in Fresh Start™
- Enzymes tested at equivent activities based on azocasein units/ml for protease and dinitrosalicylic acid units/ml for amylase.

The legend for Figure 10 is as follows:

CONDITIONS	DETERGENT TYPE	TEMPERATURE	STAIN
A	Nonphosphate	80°F	Blood/Milk/Ink
B	Nonphosphate	80°F	Chocolate Fudge Pudding
C	Nonphosphate	120°F	Blood/Milk/Ink
D	Nonphosphate	120°F	Chocolate Fudge Pudding
E	Phosphate	80°F	Blood/Milk/Ink
F	Phosphate	80°F	Chocolate Fudge Pudding
G	Phosphate	120°F	Blood/Milk/Ink
H	Phosphate	120°F	Chocolate Fudge Pudding

Even when the amount of [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]-subtilisin was used at one-third of the activity as the Novo Alcalase control, the analog of the present invention was still superior in performance in Table 6.

TABLE 6

Washing Machine Evaluation Of [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]-Subtilisin Phosphate-Containing Fresh Start™ at 100°F		
	Total delta Rd	
	Novo	143
Without Ballast*	72.6	76.6
With Ballast	44.0	56.4
Total Rd Loss Due to Ballast	28.6	20.2 ·
Novo used at 9 azo/L while 143 used at only 3 azo/L		

* Ballast refers to additional soiled clothing that was added to the washing machine in addition to soiled swatches of fabric.

Moreover, for practical reasons many industrial processes are conducted at temperatures that are above the stability temperature range of most enzymes. Therefore, although detergent applications have been emphasized herein, it is believed that subtilisin analogs according to the present invention are not only advantageous to certain industries such as detergent industry, which already require stable subtilisins, but also may be useful in industries that use chemical means to hydrolyze proteins, e.g. hydrolysis of vegetable and animal proteins.

Therefore, it is intended that the present invention include all such modifications and improvements as come within the scope of the present invention as claimed.

Claims

Claims for the following Contracting States : AT, BE, CH, LI, DE, DK, FR, GB, GR, IT, LU, NL, SE

1. A substantially pure [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin analog, optionally with one additional substitution selected from [Leu^{31}] and [Leu^{124}].

2. A composition comprising an effective amount of a subtilisin analog of Claim 1 in a detergent formulation.

Claims for the following Contracting State : ES

1. A substantially pure [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin analog, optionally with one additional substitution selected from [Leu^{31}] and [Leu^{124}].

2. A composition comprising an effective amount of a subtilisin analog of Claim 1 in a detergent formulation.

3. A process for the production of subtilisin analogs comprising recombinantly producing a subtilisin analog from a host cell having nucleic acid encoding a subtilisin analog [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}], optionally with one additional substitution selected from [Leu^{31}] and [Leu^{124}] and purifying the subtilisin analog from the fermentation broth to obtain a substantially pure [Asp^{76}, ser^{109}, Ser^{218}, Ala^{222}] subtilisin analog, optionally with one additional substitution selected from [Leu^{31}] and [Leu^{124}].

4. The use of an effective amount of a substantially pure [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] subtilisin analog, optionally with one additional substitution selected from [Leu^{31}] and [Leu^{124}] in the manufacture of a detergent composition.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, LI, DE, DK, FR, GB, GR, IT, LU, NL, SE

1. Ein im wesentlichen reines [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]-Subtilisin-Analog, fakultativ mit einer zusätzlichen Substitution, die ausgewählt ist aus [Leu^{31}] und [Leu^{124}].

2. Eine Zusammensetzung, die eine wirksame Menge eines Subtilisin-Analogs nach Anspruch 1 in einer Waschmittelformulierung umfaßt.

5

Patentansprüche für folgenden Vertragsstaat : ES

1. Ein im wesentlichen reines [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]-Subtilisin-Analog, fakultativ mit einer zusätzlichen Substitution, die ausgewählt ist aus [Leu^{31}] und [Leu^{124}].

10 2. Eine Zusammensetzung, die eine wirksame Menge eines Subtilisin-Analogs nach Anspruch 1 in einer Waschmittelformulierung umfaßt.

3. Ein Verfahren zur Herstellung von Subtilisin-Analogen, welches die rekombinante Produktion eines Subtilisin-Analogs aus einer Wirtszelle, mit einer Nucleinsäure, die ein Subtilisin-Analog [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] codiert,

15 fakultativ mit einer zusätzlichen Substitution, die ausgewählt ist aus [Leu^{31}] und [Leu^{124}], und die Reinigung des Subtilisin-Analogs aus der Fermentationsbrühe umfaßt, um ein im wesentlichen reines [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]-Subtilisin-Analog, fakultativ mit einer zusätzlichen Substitution, die ausgewählt ist aus [Leu^{31}] und [Leu^{124}], zu erhalten.

20 4. Die Verwendung einer wirksamen Menge eines im wesentlichen reinen [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}]-Subtilisin-Analogs, fakultativ mit einer zusätzlichen Substitution, die ausgewählt ist aus [Leu^{31}] und [Leu^{124}], zur Herstellung einer Waschmittelzusammensetzung.

25

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, LI, DE, DK, FR, GB, GR, IT, LU, NL, SE

30 1. Analogue de subtilisine [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] sensiblement pur, éventuellement avec une substitution supplémentaire choisie à partir de [Leu^{31}] et [Leu^{124}].

2. Composition comprenant une quantité efficace d'un analogue de subtilisine de la revendication 1 dans une formulation détergente.

35

Revendications pour l'Etat contractant suivant : ES

1. Analogue de subtilisine [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] sensiblement pur, éventuellement avec une substitution
40 supplémentaire choisie à partir de [Leu^{31}] et [Leu^{124}].

2. Composition comprenant une quantité efficace d'un analogue de subtilisine de la revendication 1 dans une formulation détergente.

45 3. Procédé pour la production d'analogues de subtilisine, comprenant la production, de façon recombinante, d'un analogue de subtilisine à partir d'une cellule hôte ayant un acide nucléique codant un analogue de subtilisine [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] éventuellement avec une substitution supplémentaire choisie à partir de [Leu^{31}] et [Leu^{124}], et la purification de l'analogue de subtilisine à partir du bouillon de fermentation pour obtenir un analogue de subtilisine [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] sensiblement pur, éventuellement avec une substitution supplémen-
50 taire choisie à partir de [Leu^{31}] et [Leu^{124}]

4. Utilisation d'une quantité efficace d'un analogue de subtilisine [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] sensiblement pur, éventuellement avec une substitution supplémentaire choisie à partir de [Leu^{31}] et [Leu^{124}] dans la fabrication d'une composition détergente.

55

FIG. I

α-aspartyl glycyl   and   β-aspartyl glycyl

2 | OH

asparaginyl glycyl   →   anhydroaspartyl glycyl   +NH₃
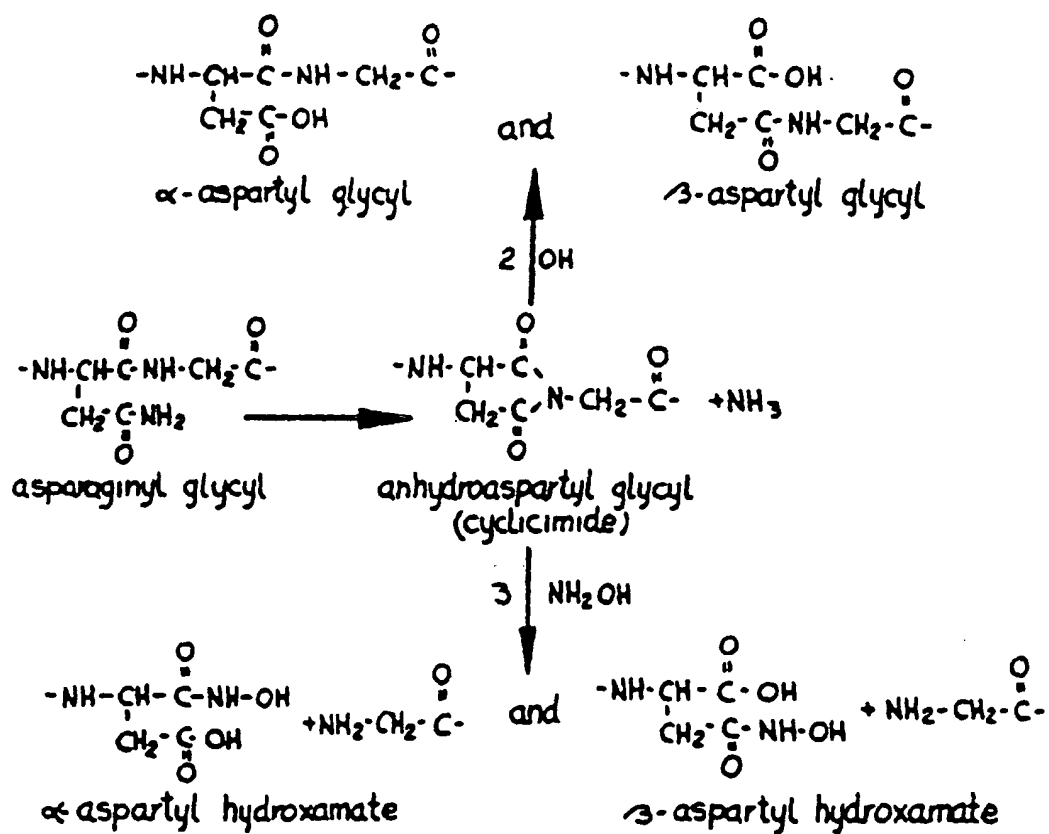   (cyclicimide)
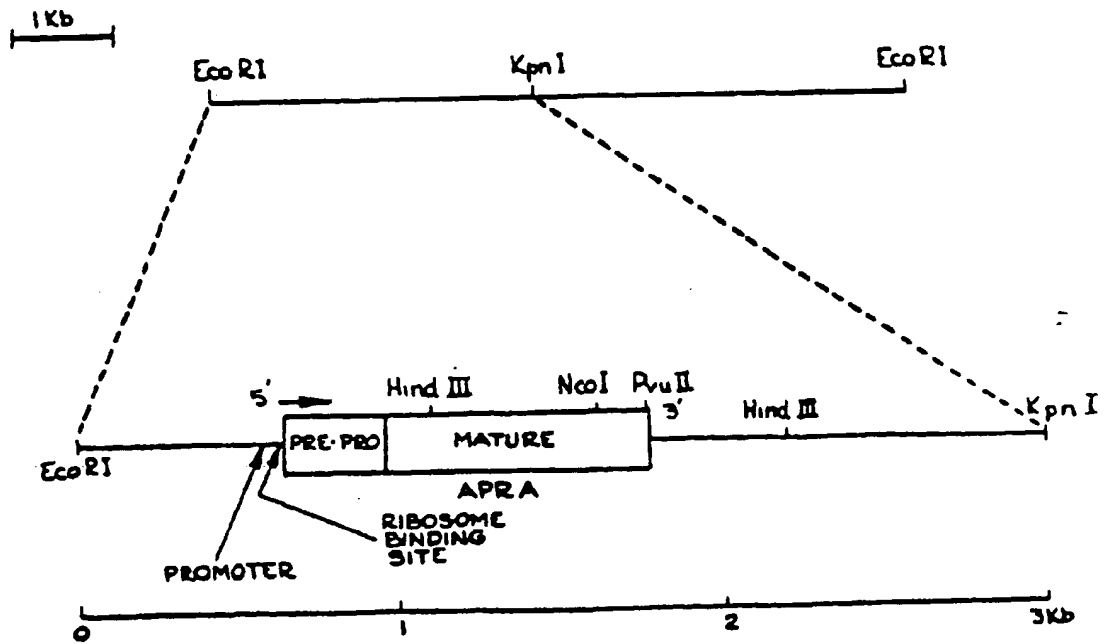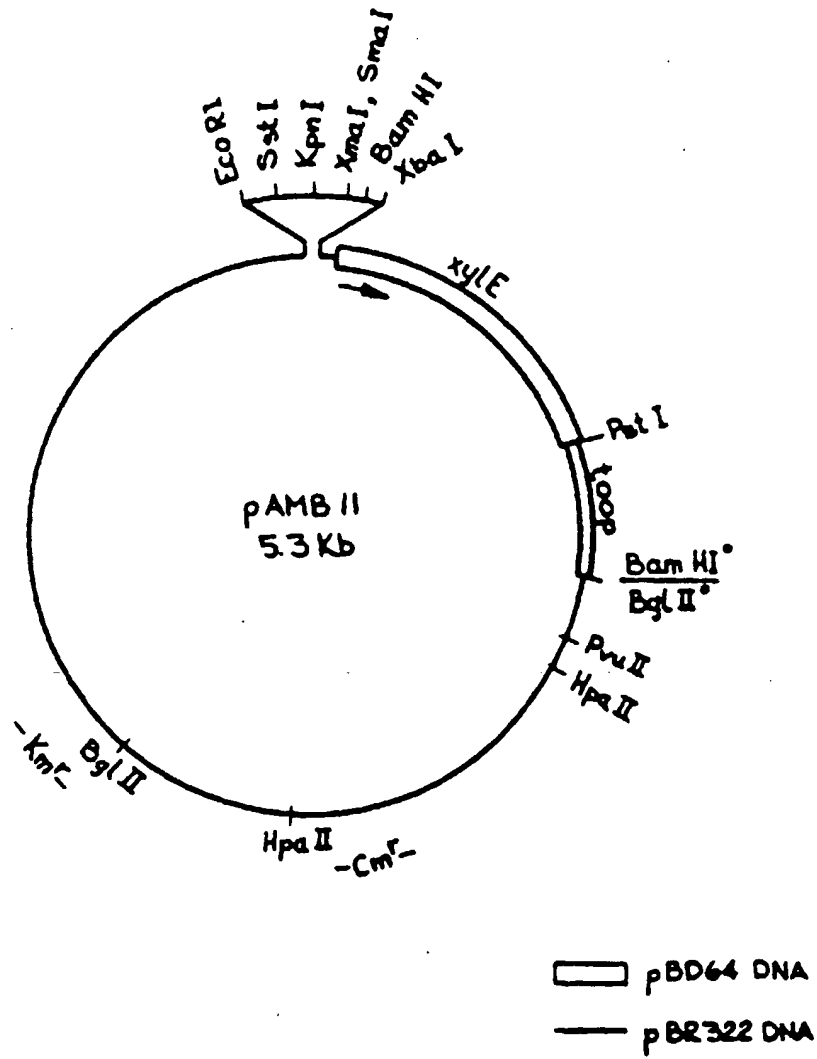
3 | NH₂OH

α aspartyl hydroxamate   and   β-aspartyl hydroxamate

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

pAMB110
7.2 Kb

EcoRI
Pre-Pro
HindⅢ
KpnI
BamHI
XbaI
xylE
λto
PstI
BglⅡ
KmR
CmR

BamHI
PstI

5' GATCTGCA 3'
T4 DNA LIGASE

pAMB106
6.2 Kb

EcoRI
Pre-Pro
HindⅢ
KpnI
λto
PstI
BglⅡ
KmR
CmR

FIG. 7

KpnI HindIII   KpnI   HindIII

• • •   'aprA   • • •

3'   5'

M13 mp18 apr2 KpnI

KpnI

T$_4$ DNA ligase

KpnI   HindIII

• • •   'aprA   • • •

3'   5'

M13 mp18 apr4

FIG. 8

Effect of 2% Chlorox at 50°C on Activity

a=[Asp^{76},Ser^{109},Ser^{218},Ala^{222}]-subtilisin and
[Asp^{76},Ser^{109},Ser^{218}]-subtilisin incubated in 2% Chlorox and
1% SDS.

b=[Asp^{76},Ser^{109},Ser^{218},Ala^{222}]-subtilisin incubated in 2% Chlorox.

c=[Asp^{76},Ser^{109},Ser^{218}]-subtilisin incubated in 2% Chlorox.

FIG. 9

WASHING PERFORMANCE, ALCALASE VS. [Asp[76], Ser[78], Ser[109], Ser[210], Ala[222]] - SUBTILISIN
IN NON-PHOSPHATE FRESH START DETERGENT, 120°F WASHING TEMPERATURE
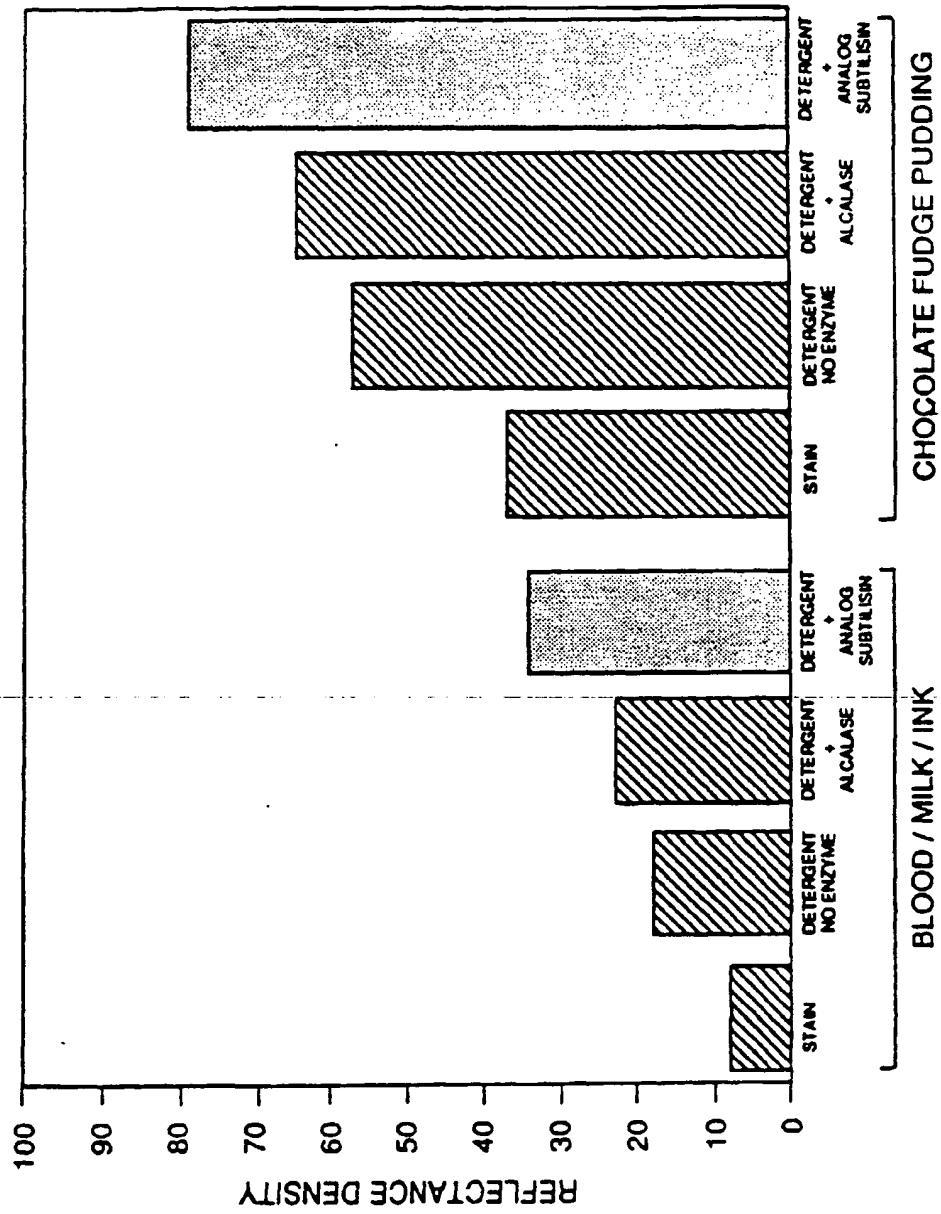
FIG. 10

WASHING PERFORMANCE: ALCALASE (1) [Asp^{76}, Ser^{109}, Ser^{218}, Ala^{222}] - SUBTILISIN (2)